FRACTIONATION OF PEPSIN-DIGESTED, DENATURED COLLAGEN

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SUMMARY

The fractionation and purification of the fragments from pepsin-digested, denatured rat-tail-tendon collagen is described. The flow sheets in Figs. 4 and 11 illustrate the two procedures which have been applied at two stages of degradation. Several fractions, which vary widely in mol. wt., could be separated and purified.

INTRODUCTION

The individual peptide chains of tropocollagen can be broken down at various levels: cleaving the telopeptides from the intact helical part¹⁻³, breaking the chains into two parts with proteolytic enzymes4-6, or to a mixture of fragments of various sizes by pepsin⁷, collagenase⁸, cyanogen bromide⁹, or hydroxylamine¹⁰ and finally to small fragments with collagenase^{11,12}, trypsin¹³ and pepsin¹⁴. The telopeptides and other small fragments can be isolated from the degradation mixtures but the breakdown products in the range of 10 000-90 000 have been difficult to deal with and the question has sometimes been dismissed because of the complexity of the fragments. This report is an attempt to face the problem. The present experiments have been performed on pepsin-treated, denatured collagen7 but the experience in the fractionation should also be applicable to other degradation mixtures of collagen.

EXPERIMENTAL

Collagen

The tail-tendon fibres of rat were dissected, rinsed several times with cold water, blotted and weighed. They were extracted with 100-fold volume (v/w) of 3% (v/v)acetic acid for 48 h at 4° with intermittent shaking. The solution was cleared by centrifugation at 35 000 \times g for 30 min in a refrigerated centrifuge and dialysed against 0.5% (v/v) acetic acid for 48 h, lyophilized and stored at 4°.

Abbreviations: S-200 etc., Sephadex G-200 etc.; CMC, carboxymethyl cellulose; PC, phosphocellulose; GF, gel-filtration chromatography; PGE, preparative gel electrophoresis; bands A-F defined in earlier publication? on starch-gel electrophoresis of pepsin-digested collagen.

Treatment with pepsin

Stage I. Collagen was dissolved overnight with gentle shaking in 0.5% (v/v) acetic acid to contain about 0.5% (w/v) of protein. The pH of the solution was adjusted to 2.0 with 1 M HCl. Pepsin (EC 3.4.4.1; DAB 6, 1:3500, Lot 741531, E. Merck A.G., Darmstadt, Germany) was added as a solid, usually to an enzyme:substrate ratio of 1:50. Crystalline preparations of pepsin were similar in effect. This mixture was divided into aliquots and stored frozen. An aliquot of the mixture was melted at about 20° and then kept for about 15 min at 40° and used immediately for the fractionations. The kinetics of the breakdown have been studied separately¹⁵. Under controlled conditions the qualitative starch-gel electrophoretic pattern of the digest was reproducible. Control experiments confirmed that in the cold room the starch-gel electrophoretic pattern remained constant and control collagen intact for 24 h.

Stage II. The enzyme treatment lasted 24 h at 38°. As a rule, the enzyme: substrate ratio of 1:100 was used.

Gel-filtration chromatography

Sephadex G-200 column. The dextran gel (Lot No. To-6471 from Pharmacia Fine Chemicals, Uppsala, Sweden) was allowed to swell for 4 days in 0.5% (v/v) acetic acid. The size of the column was first 3 cm \times 60 cm and later 4.5 cm \times 90 cm. In the larger column 10% of cellulose powder (Whatman Column Chromedia CG 11) was added.

The usual sample contained about 100 mg of protein in 20 ml of solution. The column was eluted with 0.5% acetic acid at 4°. The flow rate was initially 20 ml/h but slowed down to 10 ml/h when the column became more densely packed. Three to five runs were carried out with the same column. The fractions were collected at 15–20-min intervals. Biuret reaction was carried out from suitable aliquots and the fractions pooled as shown in Fig. 2 and lyophilized.

Experiments were also carried out with 0.2% HCl or 0.2% formic acid as the eluant. The resolution was not better than with acetic acid. Some runs both with HCl and acetic acid were performed at room temperature and at 40°. At 40° the Fractions S-IV-VII increased.

The rechromatography experiments were carried out at room temperature with a column of 3.5 cm \times 90 cm.

Other Sephadex columns. The Sephadex materials (G-10, Lot No. To-7929, G-25F, Lot No. To-5547F, G-50F, Lot No. To-4884 F, G-75M, Lot No. To-8492M) were allowed to swell for 24–48 h and then allowed to settle into a column of 1.1 cm \times 46 cm at 40°. Samples of at least 0.2 mg were analysed. The eluant was pyridine–acetate solution (10 ml of pyridine, Baker's Analysed, Lot No. A 0108, and 9 ml of acetic acid were dissolved in water and filled to 2000 ml), which was pumped through the column at 3 ml/h and 1-ml fractions were collected. Blue dextran (Pharmacia) was used as a marker of the void volume. The emergence of salts was detected with AgNO₃ solution.

DEAE-Sephadex and CM-Sephadex were also tried for the fractionation of the digestion mixtures. The results were not encouraging and the details are not given here.

Desalting. The lyophilized material (Stage II) was dissolved in pyridine–acetate which was allowed to drain into the Sephadex G-25 column 2.3 cm \times 31 cm, which was kept at 40°, subsequently eluted (60 ml/h) with the same solvent and 5-ml fractions were collected. In the work on the degradation mixture at Stage I, the column was eluted with 0.5% (v/v) acetic acid at room temperature.

Recycling chromatography. The column was 3.5 cm × 90 cm (LKB-Produkter, Stockholm, Sweden), filled with Sephadex G-200. Samples of 75-100 mg were dissolved in 10-20 ml of 0.5% acetic acid which was also used as eluant. The procedure was found useful only in the refractionation of the combined Peaks S-200-(I-III) from the Sephadex G-200 column.

Starch-gel electrophoresis

Analytical procedure. The equipment and method have been described separately^{17,18}. Acetate buffer, pH 4.7, I = 0.017, was used. The temperature was 37°, duration 4-6 h, voltage, 120 V, and the concentration of the gel (Lot 225-1, Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada) 14.75% (w/v). The sheets were stained with 0.1% nigrosine in a boiling-water bath for 30 min and the excess nigrosine washed in 50% (v/v) glycerol.

Preparative starch-gel electrophoresis. The method and equipment have been described separately19. The length of the separation column varied from 6 to 12 cm, the pH of the buffer was 4.2, and the elution rate 60–80 ml/h for 12–18 h. About 10-ml

fractions were taken. The sample contained 25 mg of protein maximally.

Preparative elution of collagen from starch-gel strips. A 5-mg sample was imbibed in a strip of 1.4 cm \times 6.0 cm of Whatman 3 MM paper, which was placed in a slot of a usual electrophoretic gel sheet. Sodium acetate buffer, pH 4.7, and a voltage of 120 V were used. After a run of 6 h the strips were stained on the surface. The bands were cut from the bulk of the sheets, pooled and minced on the separation gel in the column electrophoretic assembly. Each fraction was driven electrophoretically to the elution chamber. The yields were so small that the method was not practicable. Only the α' fragment could be obtained in reasonable yield.

Fractionation with ion-exchange cellulose columns

Phosphocellulose. The cation exchange material (Whatman P-70) was suspended overnight in water, and washed in a funnel with 0.5 M NaOH, water, 1 M HCl, water and finally with 0.001 M acetate buffer, pH 3.8. A column on 1.1 cm imes 16 cm was used and eluted at room temperature with a linear gradient20, arranged with combined vessels (820 + 820 ml), which contained, in the beginning, acetate buffer and 0.6 M NaCl in the same buffer, respectively. The elution rate was 70-90 ml/h (later 25-30 ml/h) and 5-ml fractions were collected. Samples contained 50–100 mg protein in 5 ml of solution.

Carboxymethyl cellulose. The material (Whatman CM-70) was allowed to swell in the initial buffer, then washed with o.or M NaOH and o.5 M NaCl. The columns (3 cm imes 18 cm or 2 cm imes 20 cm) were packed with small portions of the slurry applying N_2 pressure (0.5 kg/cm²). The temperature in the jacket was 40°. A linear gradient at pH 4.2, ionic strength rising from 0.06 to 0.16 or 0.20, was arranged with NaCl in acetate buffer. The elution rate was 40 ml/h and 8-ml fractions were taken. The size of the sample was 100 mg of collagen in 20 ml of solution.

Analytical methods

The location of the collagen fractions was performed in most cases with a modified biuret reaction16. In some experiments the absorption at 2300 Å was measured, applying flow cuvette and Hitachi-Perkin Elmer Model 139 spectrophotometer.

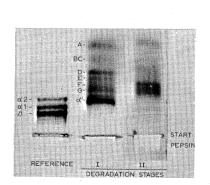
The ninhydrin reaction was carried out with a modification of the conventional procedure²¹.

RESULTS

Initial fractionation

Earlier work¹⁵ has shown that the initial phase of the degradation (referred to here as Stage I) is rather reproducible, and that further digestion first occurs after a longer interval (Stage II). Samples representing these two stages (Fig. 1) were chosen for the exploratory fractionation.

In spite of several attempts made with various methods (applying preparative gel electrophoresis (PGE), gel filtration and recycling chromatography with Sephadex columns, ion-exchange chromatography with Dowex 50, phospho- and CM-cellulose, CM- and DEAE-Sephadex columns) no single method was found to be sufficient for



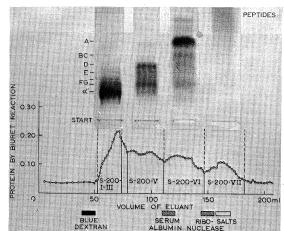


Fig. 1. Starch-gel electrophoretic patterns of denatured collagen degraded with pepsin to Stages I and II. The designation of the bands as in the refs. 7 and 15. The scheme of the fractionation of pepsin-degraded collagen is shown in Fig. 4 for Stage I and in Fig. 11 for Stage II.

Fig. 2. Fractionation of pepsin-degraded collagen (Stage I) with Sephadex G-200 column. Designations of the peaks refer to Fig. 4. The respective analytical starch-gel electrophoretic patterns are included.

the complete resolution of either pepsin digest of collagen. It was necessary to work in steps and select for a preliminary fractionation some simple method of large capacity.

For the Stage I digest, the appropriate method was found to be a discontinuous fractionation with Sephadex G-200 column (Fig. 2). The typical pattern was quite reproducible. The various bands⁷ revealed by starch-gel electrophoresis were enriched from Peaks S-200-I to VI, but S-200-VII was composed of peptides so small that they leached from the gel sheet. The size of the Fraction S-200-VII increased with longer digestion at the expense of the other fractions. Pepsin emerges in Peaks S-200-VI and VII.

In comparison with serum albumin and ribonuclease, most of the collagenous

fragments were eluted quite rapidly from the Sephadex G-200 column (Table I). The various methods for the estimation of the mol. wts. from gel-filtration do not seem to be applicable to collagen fragments.

When the digestion had proceeded further (Stage II), the CM-cellulose fractionation was more useful for the initial separation. In Fig. 3 is shown the transition of the chromatographic pattern from Stage I to II. The corresponding gel-electrophoretic

TABLE I MIGRATION RATES OF THE VARIOUS COLLAGEN FRACTIONS IN SEPHADEX COLUMNS K_d defined according to Flodin²⁶. The sum of the imbibed and void volumes was determined from the emergence of salts.

Sample	Sephadex	K _d
Stage I:		
S-200- (I-III)	G-200	0.2
S-200-IV	G-200	0.3
	G-75M	O.I
S-200-V	G-200	0.5
	G-75M	0.1
S-200-VI	G-200	0.7
	G-75M	0.2, 0.
Serum albumin	G-200	0.6
Ribonuclease	G-200	0.9
Stage II		
CMC-11/S-75-1	G-75M	0.5
CMC-II/S-75-2	G-75M	0.2
CMC-II/S-75-3,4	G-75M	0.0
CMC-III/PGE-1	G-75M	0.9
	G-50F	0.0
CMC-III/PGE-2	G-50F	I.I
	G-25F	0.0
CMC-(IV-VI)/PGE-1	G-25F	1.3
	G-10	O.I
CMC-(IV-VI)/PGE-2	G-75M	1.2
	G-50F	-0.1
CMC-(IV-VI)/PGE-3/S-50-1	G-200	0.3
	G-75M	0.0
CMC-(IV-VI)/PGE-3/S-50-2	G-50F	approx. 1.0
	G-25F	/ I.4
ar component	G-200	0.0

patterns of Stage II are shown in Fig. 12. Fraction CMC-I contains small peptides and pepsin. The bands are heterogenous, but the separation of the components could be worked out.

Purification of the fractions obtained from the Stage I digest (summarized in Fig. 4)

Purification of the fragment α' from the Fraction S-200-(I-III). The material contains original α components, but in addition a fragment which has not previously been recognized and is designated here as α' . It migrates on starch-gel electrophoresis between the α I and α 2 components depending on the ionic strength and pH. It could be purified with a good yield by rechromatography with Sephadex G-200 column (Fig. 5).

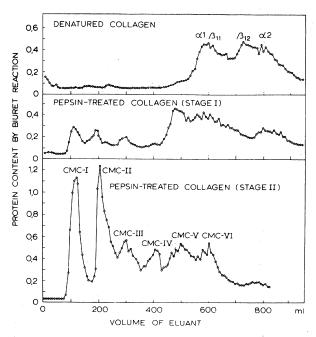


Fig. 3. Fractionation patterns of pepsin-degraded collagens by the elution of CM-cellulose column. For the samples the reader is referred to Fig. 1, for the designations of the peaks (Stage II), to Fig. 11, and for the starch-gel electrophoretic patterns, to Fig. 12. The control sample without pepsin had been kept at pH 1.7 at 38° for 3 h. In this case Stage I samples were digested for 3 h, those of Stage II for 24 h. The enzyme:substrate ratio was 1:100.

Separation and purification of the materials in the electrophoretic bands D-G from the Fraction S-200-V. The best method for the further analysis of S-200-(IV, V) was CM-cellulose chromatography. Fig. 6 shows how three fractions, at least could be obtained. From the electrophoretic patterns of the various CM-cellulose fractions it

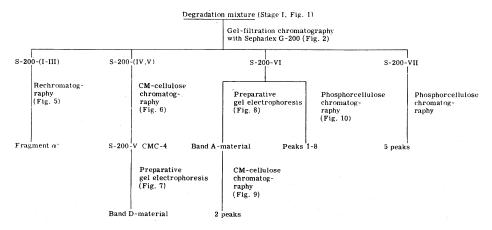


Fig. 4. Flow sheet on the fractionation of pepsin-degraded collagen, Stage I (cf. Figs. 1 and 3).

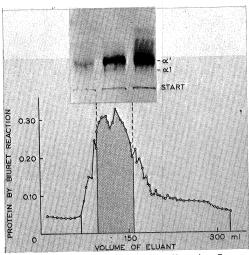
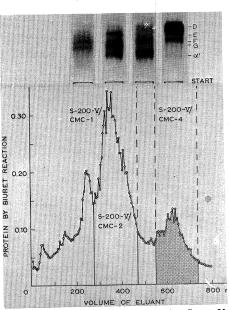


Fig. 5. Rechromatography of the Fraction S-200-(I-III) in Sephadex G-200 column for the purification of the fragment α'. The respective analytical electrophoretic patterns are included.

is apparent that the Peak S-200-V/CMC-4 in Fig. 6 is the same as the band D material in the original gel-electrophoretic pattern. This fraction could be purified further by preparative starch-gel electrophoresis (Fig. 7).

The separation of the band E-G materials S-200-V/CMC-1 and 2 could not be



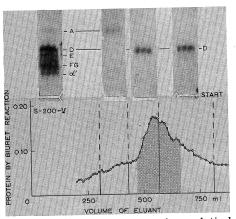


Fig. 6. Fractionation of the Fraction S-200-V with CM-cellulose column. The respective analytical electrophoretic patterns are included (cf. Figs. 1 and 4).

Fig. 7. Purification of the Fraction S-200-V/CMC-4 (the shaded area in Fig. 6) with preparative starch-gel electrophoresis. The respective analytical patterns are included, with the Fraction S-200-V on the left.

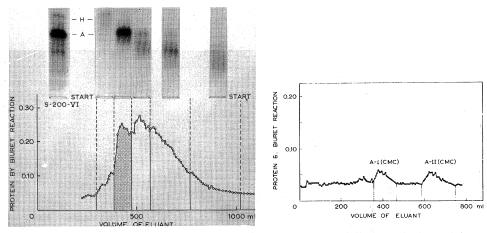


Fig. 8. Purification of the Fraction S-200-VI with preparative starch-gel electrophoresis. The shaded area contains the material migrating as band A in analytical starch-gel electrophoresis. The pattern of the unfractionated sample on the left.

Fig. 9. The division of purified S-200-VI (band A material) to two fractions by CM-cellulose column chromatography.

accomplished in spite of much effort. Various Sephadex columns operated both with recycling and discontinuously, elution from starch-gel strips and preparative starch-gel electrophoresis were tried, all with unsatisfactory results. One reason for this failure is that the fragments decompose during the manipulations.

Separation and purification of the fragments from the Fraction S-200-VI. This bulky fraction was found to be heterogeneous. Applying the preparative starch-gel electrophoresis some fractionation was apparent and one of the fractions (shaded in Fig. 8) was found to correspond to band A. When this fraction was subjected to CM-cellulose fractionation, it was divided into two subfractions, A-I and A-II (Fig. 9).

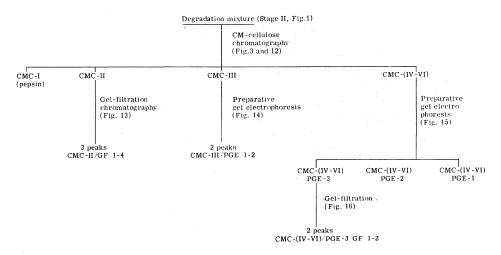


Fig. 11. Flow sheet on the fractionation of pepsin-treated collagen, Stage II (Figs. 1 and 3).

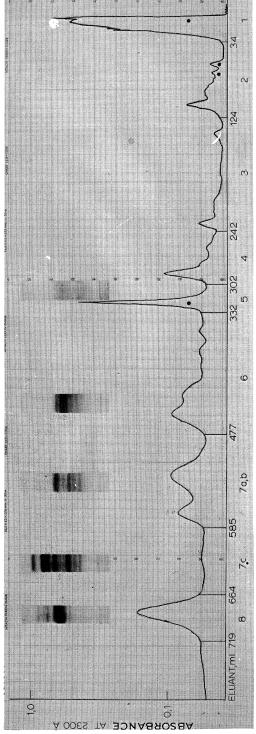
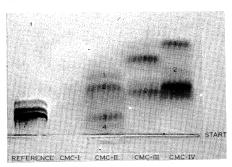


Fig. 10. Refractionation of the Fraction S-200-VI by PC column chromatography. The analytical electrophoretic patterns of the materials are included. Speed of the paper, 23.4 mm/h; elution rate, 25 ml/h. The peaks marked with dots contain material originating from the pepsin preparation.



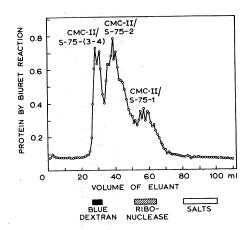


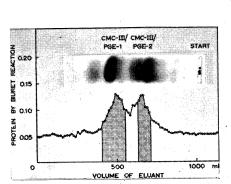
Fig. 12. Analytical starch-gel electrophoretic patterns of the fractions of pepsin-degraded collagen, Stage II (Fig. 1, right, and Fig. 3, bottom), obtained by CM-cellulose chromatography.

Fig. 13. Separation of the fragments of CMC-II (Figs. 3 and 12) by gel filtration with Sephadex G-75 M column. The flow was 1.5 ml/h and 0.5-ml fractions were collected.

The use of phosphocellulose (PC) column for the Fraction S-200-VI was rewarding (Fig. 10). The heterogeneous nature of band A material is apparent again. Peaks 6-8 contain different band A materials, derived from Fraction S-200-VI, while Peaks 1-5 contain material derived from S-200-VII.

Fractionation of the peptide mixture from the Fraction S-200-VII. This material was divided by PC column chromatography into several sharply resolved fractions which contain peptides and other substances of small mol. wt. These fractions will be described separately.

Remarks. The various Sephadex G-200 column fractions were studied systematically with various Sephadex columns (Table I) but no more resolution was obtained except that the Fraction S-200-VI was divided into two peaks, which may depend on



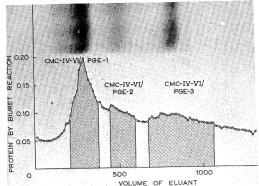


Fig. 14. Separation of the fragments from CMC-III (Figs. 3 and 12) by preparative starch-gel electrophoresis. The analytical starch-gel electrophoretic pattern is included.

Fig. 15. Separation of the fragments from CMC-(IV-VI) (Figs. 3 and 12) by preparative starch-gel electrophoresis. The analytical starch-gel electrophoretic pattern is included.

impurities. PC column was not helpful in the study of fragments other than S-200-VI and VII. CM-cellulose column had the drawback that much material was retained in the column under conditions in which the various α and β components are eluted.

Preparative work on collagen degraded with pepsin to Stage II (summarized in Fig. 11)

The fractions illustrated in Fig. 3 (bottom) are designated CMC-I to VI, but CMC-I did not contain any material which could be demonstrated by analytical starch-gel electrophoresis (Fig. 12).

The three main fractions in CMC-II could be separated rather well with a Sephadex G-75 column (Fig. 13). CMC-III also could be divided into several subfractions with preparative gel electrophoresis (Fig. 14).

The Peaks CMC-(IV–VI) were treated as a pooled sample which could be divided into three subfractions by preparative starch-gel electrophoresis, as shown in Fig. 15. The fraction CMC-(IV–VI)/PGE-3 was heterogeneous and it was divided further into two fractions with a Sephadex G-5oF column (Fig. 16). As judged from the behaviour in various Sephadex columns, the mol. wt. of the Fragment CMC-(IV–VI)/PGE-3/S-50-I is in the range of 50 000–100 000, which was confirmed by comparison with a purified α I component (Table I).

The mol. wts. of the fragments differ over a very wide range from some thousands to several tens of thousands. Courts¹⁴ found an average mol. wt. in pepsin-degraded gelatin of 10 000.

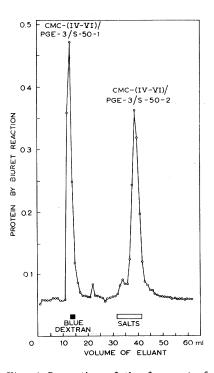


Fig. 16. Separation of the fragments from CMC-(IV-VI)/PGE-3 (Fig. 15) with gel filtration applying Sephadex G-50.

The patterns obtained from the αI and $\alpha 2$ components are not similar. This may reflect the different amino acid compositions or rather difference in the stabilities between the chains (unpublished work).

The fractionation of the breakdown mixture at Stage I is more difficult but also more rewarding, because the fragments are larger, on the average, than in the State II mixture. It is gratifying that pepsin is removed in the initial fractionations so that it does not complicate the later procedures.

The complete fractionation of the pepsin digests would be a tedious and perhaps useless work. Such fractions as could be purified with a reasonable effort and yield, however, would be useful as the basis of structural and comparative work. As examples one could mention the fragment α' from S-200-(I–III), band D material from S-200-V/CMC-4, various band A materials from S-200-VI and the prominent peaks from S-200-VI and VII.

Of special importance are the large fragments. Thus far they have been made available by treatment of native collagen with pepsin^{3,13} and other proteolytic enzymes^{5,6,8}. From the present fragments, α' and CMC-(IV-VI)/PGE-3/S-50-1 belong to that category, as do the band E-G materials which could not be obtained pure.

The Fraction S-200-(I–III) should contain intermolecular aggregates and non-collagenous macromolecules (proteins and carbohydrates) associated with the quaternary structure of collagen. Fraction S-200-VII contains the telopeptides but also material from the helical part of the molecule. It could also be expected to contain peptide fragments bound together by linkages analogous to those which form β components from α components¹¹.

The sensitivity of the various linkages to pepsin is very variable²² and there is evidence that the first cleavage occurs adjacent to aspartic and glutamic acids. The starch-gel electrophoretic pattern of the breakdown mixtures of collagens from other species is quite different, as is that of the cyanogen bromide-degraded collagen²³.

The small fragments in Fraction S-200-VII emerge from the PC column in order of decreasing acidity. They all contain hydroxyproline (20–40 residues/1000) but the low content of glycine (150–220 residues/1000) indicates that only a part is derived from the helical part of tropocollagen. These small fractions in S-200-VI and VII are 2- to 3-fold richer in reactive carbonyl groups than the bulk (S-200-I to V).

The characterization of the large fragments will be published separately. The mol. wt. of fragment α' seems to be on the order of 60 000 and that of the band D material about 30 000 (ref. 24).

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